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Association of Vpu-Binding Protein with Microtubules and Vpu-Dependent Redistribution of HIV-1 Gag Protein

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The efficient exit of HIV-1 particles from cells requires the action of the viral encoded protein Vpu. Vpu-binding protein (Ubp) is a cellular protein that interacts with both Vpu and the major structural component of the viral capsid (Gag) and appears to affect the efficiency of particle exit. Elucidation of the function of Ubp and characterization of the spatial distribution of Ubp may provide information pertinent to understanding the role of Ubp in virus replication. To investigate the subcellular location of Ubp, and to see whether Vpu affects the intracellular distribution of Gag, we carried out immunofluorescence localization in conjunction with confocal microscopy. Based on this analysis Ubp is present in both the nucleus and the cytoplasm. In the cytoplasm, Ubp appeared to be associated with microtubules as evidenced by cofluorescence with tubulin in the absence and in the presence of colchicine. However, cytoskeletal isolation and detergent extraction of cells resulted in association of Ubp with the soluble fractions, indicating that Ubp is not in tight association with microtubules. Moreover, flotation gradient analysis demonstrated that Ubp is cytoplasmic and not stably associated with the plasma membrane. Interestingly, expression of Vpu in cells resulted in redistribution of both Ubp and Gag to a location near the periphery of the cell. The effect of Vpu on both Ubp and Gag protein has implications for Vpu-mediated particle exit from cells.

INTRODUCTION

Retroviral Gag protein is synthesized in the cytoplasm of virus-infected cells and associates with the cytoplasmic side of the plasma membrane by way of a myristylated N-terminal membrane-binding domain (reviewed in Swanstrom and Wills, 1997). Association with the plasma membrane and intermolecular Gag–Gag interaction result in the coalescence of approximately 1500 Gag molecules into individual viral capsids (Vogt and Simon, 1999). Successful budding of retrovirus particles from the plasma membrane requires a late domain (L domain) that is intrinsic to Gag and that is required for functional association of ubiquitina with Gag (Ott *et al.,* 1998; Patnaik *et al.,* 2000; Schubert *et al.,* 2000; Strack *et al.,* 2000). In the case of the lentivirus HIV-1, the action of the viral protein U (Vpu) is also required for efficient budding (Klimkait *et al.,* 1990; Terwilliger *et al.,* 1989).

Vpu is a 16-kDa type I integral membrane phosphoprotein encoded by the HIV-1 accessory gene *vpu* (Cohen *et al.,* 1988; Strebel *et al.,* 1988). In addition to facilitating particle exit, Vpu promotes the specific degradation of the viral receptor, CD4 (Bour *et al.,* 1995; Schubert *et al.,* 1992).

Ubp (viral protein U-binding protein), is a 34-kDa human cellular protein that binds to HIV-1 Vpu based on protein–protein interactions in a yeast GAL4 two-hybrid screen (Geraghty *et al.,* 1994) and association *in vitro* and *in vivo* (Callahan *et al.,* 1998). In addition to its interaction with Vpu, Ubp associates with HIV-1 Gag protein. Gag is the principal structural component of the viral core. Two lines of evidence are consistent with functional interaction between these viral proteins and Ubp. First, overexpression of Ubp in HIV-1 producing cells significantly reduces the amount of virus released from the cell, and second, *in vivo* association of Ubp with Gag is abrogated when Vpu is expressed in the cell (Callahan *et al.,* 1998).

Independent experiments indicate that Ubp also interacts with proteins of the parvovirus family (Cziepluch *et al.,* 1998). Ubp, designated SGT in that report (for small glutamine-rich tetratricopeptide repeat containing protein), interacts with the nonstructural viral protein, NS1, of parvovirus H1. NS1 is a nuclear protein that functions in parvovirus DNA replication and transcriptional gene expression. Thus, if Ubp/SGT affects NS1 function, that interaction may occur in the nucleus of parvovirus-infected cells. In contrast, Vpu and Gag are membrane-associated and cytoplasmic proteins, respectively. Therefore, cytoplasmic Ubp–Vpu and Ubp–Gag interaction is more relevant for HIV-1.

Ubp contains four tetratricopeptide repeat sequences

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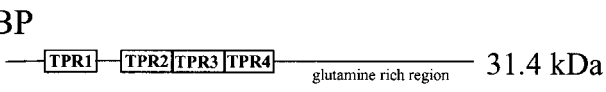

FIG. 1. Structure of Ubp and TPR regions. Schematic representation of Ubp and the location of the four tetratricopeptide repeat domains of Ubp (boxes).

(TPRs), one located near the amino-terminus and three in tandem array beginning 41 residues from the end of the first TPR (see Fig. 1). TPR motifs are found in proteins from a wide range of organisms and are present in diverse proteins that have an array of cellular functions. TPR motifs are a specific class of amphipathic alpha-helices mediating inter- or intramolecular protein–protein interaction in a "lock and key" type fashion (Goebl and Yanagida, 1991; Lamb *et al.,* 1995; Sikorski *et al.,* 1991; van der Voorn and Ploegh, 1992). Thus, the four TPR motifs of Ubp may promote protein–protein association. In Ubp, the carboxyl 118 residues, starting after the fourth TPR, constitute a glutamine-rich sequence with glutamine comprising 13% of the amino acid residues. This region of the protein contains no significant homology, based on BLAST search analysis, with any known protein sequence, with the exception of apparent *Caenorhabditis elegans, Saccharomyces cerevisiae,* and *Drosophila melanogaster* Ubp homologs. Moreover, this 118-residue stretch lacks obvious conserved domains as evidenced by comparison to the Conserved Domain Database of RPS-Blast. The only obvious segments of Ubp that contain homology to characterized proteins reside exclusively in the TPR regions. Ubp readily self-associates in an *in vitro* binding system (Cziepluch *et al.,* 1998; and Handley, unpublished data). However, the exact subunit composition of the oligomer is unknown.

The purpose of this study was to characterize the subcellular location of Ubp and Gag in the cell and compare that intracellular distribution in the presence and in the absence of Vpu. We chose to study Ubp in HeLa cells since these cells have been used extensively to examine both Vpu-mediated CD4 degradation and HIV-1 particle release, and they are large, easy to visualize cells and contain a clearly discernible cytoplasm. The results presented here indicate that cytoplasmic Ubp is associated with microtubules. Moreover, expression of Vpu in cells results in the redistribution of both Ubp and Gag to areas in close proximity with the cell periphery. The data are consistent with the possibility that Ubp is part of a cytoskeletal-associated protein complex that is affected by Vpu expression.

RESULTS AND DISCUSSION

Ubp colocalizes with cytoplasmic microtubules

Preliminary analysis of cells by immunofluorescence with anti-Ubp antibody and confocal microscopy indicated that Ubp is present in the nucleus and in association with the cytoplasmic cytoskeleton. Ubp interacts with the HIV-1 Vpu and Gag proteins. Since both of these viral proteins reside in the cytoplasm of the infected cells, and since virus assembly and budding are processes that take place in the cytoplasm and at the plasma membrane, we focused on the location of Ubp in the cytoplasm. Thus, we looked for colocalization of Ubp with either actin filaments or microtubules since these are major constituents of the cytoskeleton. Dual staining for Ubp and tubulin, or with actin, showed apparent cofluorescence of Ubp with microtubules and to a lesser extent with actin filaments (Fig. 2A). The pattern for Ubp staining in the cytoplasm is shown at higher magnification in Fig. 2B. The image shows Ubp to be localized in a filamentous branching pattern in close association with bundles of microtubules. Further, the association of Ubp with microtubules was observed in image sections taken from successive parallel planes through the cell (Fig. 3A).

If Ubp is associated with microtubules, depolymerization of microtubules might be expected to result in concomitant redistribution of cytoplasmic Ubp. To test this hypothesis, we treated cells with colchicine, a microtubule destabilizing agent. As shown in Fig. 3B, colchicine treatment resulted in the expected depolymerization of microtubules and redistribution of apparent tubulin subunits to cytoplasmic locations both adjacent to the nucleus and toward the distal regions of the cytoplasm. Similarly, the cytoplasmic Ubp in these cells was also changed from association with filamentous branching networks to positions coincident with tubulin. Additionally, Ubp appeared to maintain colocalization with tubulin in the area surrounding the nucleus as well as in the outer cytoplasmic regions of the cells. Nuclear Ubp remained apparently unchanged in the colchicine-treated cells. These data are consistent with the association between Ubp and cytoplasmic microtubules.

Cell division results in transient depolymerization and reestablishment of cytoplasmic microtubules. Thus, we thought it might be informative to examine the intracytoplasmic disposition of Ubp in dividing cells. Figure 3C depicts Ubp and forming microtubules in newly forming daughter cells. Interestingly, in these cells Ubp appears in what seems to be a globular array and without apparent association with microtubules. The presence of this spherical, microtubule-independent Ubp in dividing cells may indicate that Ubp dissociates from microtubules during cell division forming large aggregates in the cytoplasm. Since Ubp is cytoskeletally associated in nondividing cells, Ubp presumably reassociates with microtubules after formation of the two daughter cells.

To further examine the intracellular distribution of Ubp we prepared cytoplasmic and nuclear fractions and attempted to detect Ubp using Western analysis. As shown in Fig. 4A, Ubp was found in both the cytoplasmic and the

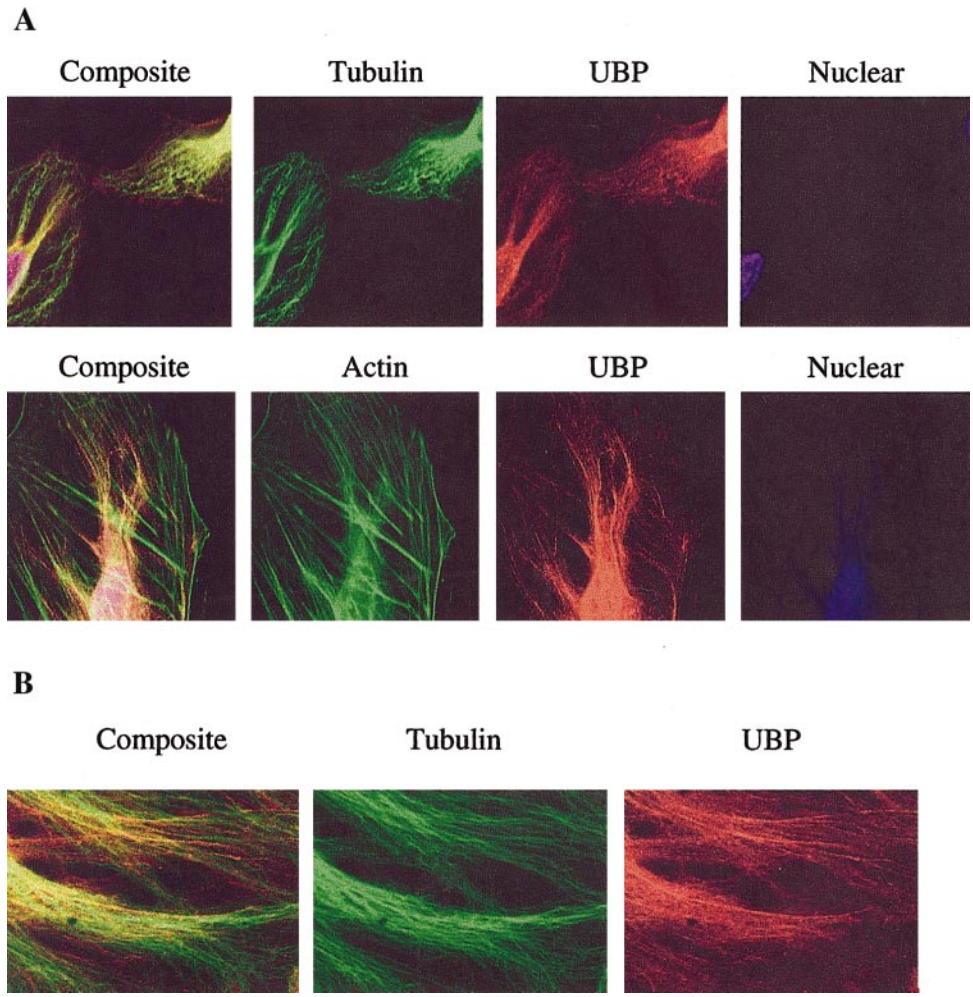

FIG. 2. Ubp colocalization with microtubules. (A) HeLa cells were fixed and stained with nuclear stain and with antibodies to Ubp and tubulin. (Top) Comparison of Ubp and tubulin in the cytoplasm. Tubulin is shown in green and Ubp is shown in red. In the composite panel, yellow indicates overlap of both tubulin and Ubp. (Bottom) Comparison of Ubp and actin filaments. (B) Higher magnification image of Ubp and microtubules in the cytoplasm of the cell.

nuclear fractions, although the protein appeared to be somewhat more abundant in the cytoplasm than in the nucleus. This is consistent with the immunofluorescence and confocal analyses of Ubp.

Cytoplasmic Ubp is not tightly associated with microtubules

To further explore the interaction between Ubp and microtubules, we prepared cytoplasmic and nuclear fractions and carried out cytoskeletal fractionation of cells. For cytoskeletal fractionation, microtubules and actin filaments were stabilized in buffer containing taxol and phalloidin, extracted from cells, washed, solubilized, and analyzed by Western blotting (see Materials and Methods). This fractionation procedure results in a combined cytoplasmic and soluble membrane fraction and a separate cytoskeletal fraction. These fractions were analyzed using antibody specific for Ubp, tubulin, actin, kinesin, and β-COP. The kinesins are a family of microtubule-based motor proteins (Hackney and Jiang, 2001; Vale and Milligan, 2000) and detection of kinesin served as a representative control protein that is tightly associated with microtubules. β-COP (cotamer) is a protein that helps mediate intracellular membrane trafficking (Contreras *et al.,* 2000; Tisdale, 2000); we used β-COP as a representative control protein associated with cytoplasmic intracellular membranes and as a protein that is not stably associated with the cytoskeleton.

As shown in Fig. 4B, cytoskeletal fractionation revealed that Ubp was exclusively found in the cytoplasmic fraction (S) and not in the cytoskeletal fraction (I). We also carried out coimmunoprecipitation experiments using initial immunoprecipitation with anti-Ubp and anti-tubulin antibody and subsequent Western analysis of the immunoprecipitate with anti-tubulin and anti-Ubp antibody, respectively. In both protocols, there was no detectable stable association between Ubp and tubulin (data not shown). Taken together, these results indicate that the

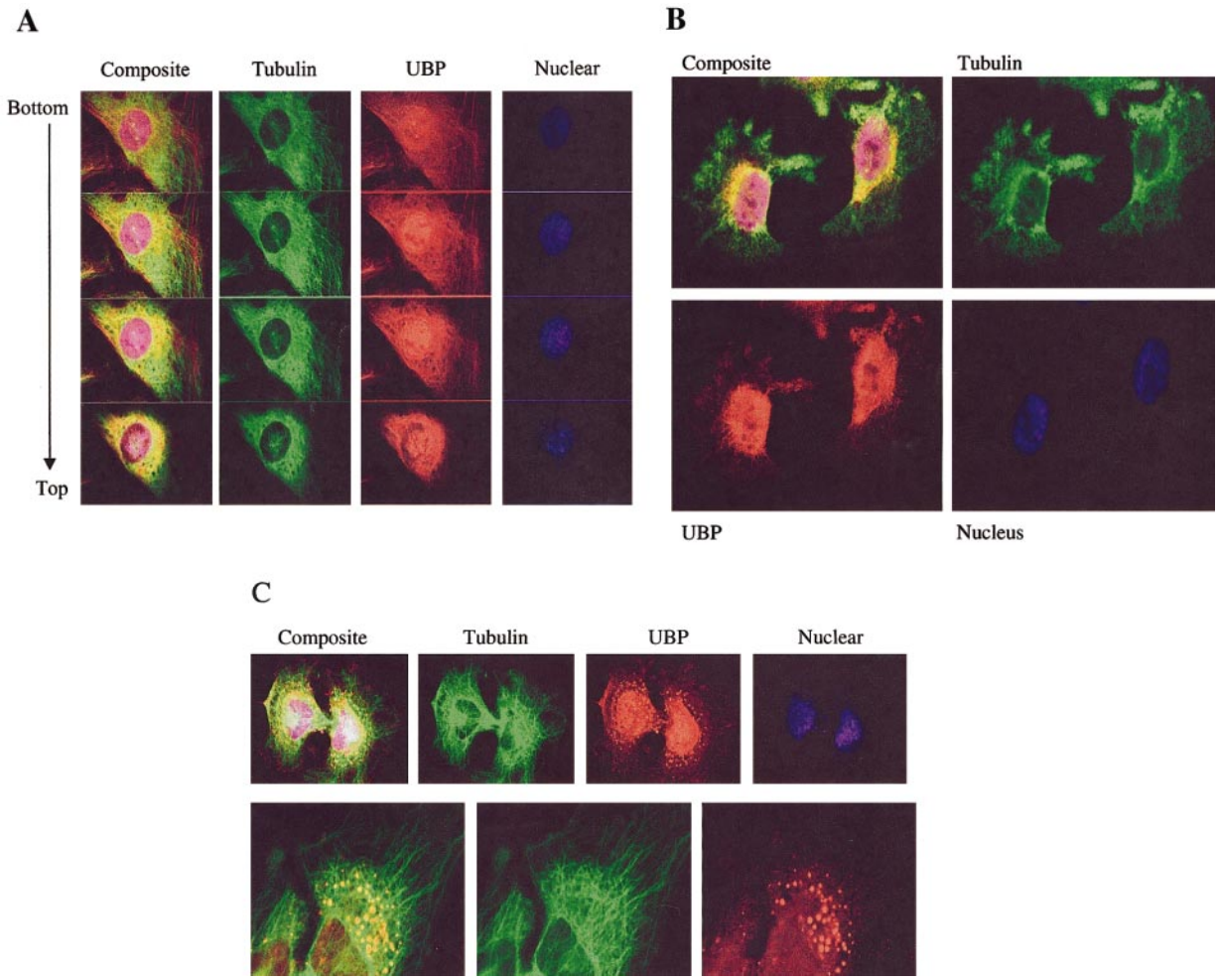

FIG. 3. Cytoplasmic Ubp and tubulin association. (A) Sections of cells stained for tubulin, Ubp, and nuclei. The top panel shows the bottom of the cells with sections moving up through the cells. (B) Altered Ubp location of Ubp with colchicine treatment. Immunofluorescence staining of Ubp and tubulin of colchicine-treated HeLa cells. (C) Cytoplasmic distribution of Ubp in dividing cells. The top panel of images shows two daughter cells that are at the point of separating. The bottom panel shows a higher magnification view of the cytoplasm of a forming daughter cell. Nuclear staining was not carried out for this set of images.

apparent Ubp association with microtubules as evidenced by confocal imaging is not a direct association or is a weak interaction.

Ubp is not stably associated with cellular membranes

Vpu is associated with cellular membranes by way of an N-terminal hydrophobic membrane anchor. Gag becomes associated with the cytoplasmic face of the membrane by way of the "M domain." Ubp does not have a contiguous hydrophobic domain that is a candidate that mediates Ubp association with membranes. However, since Ubp interacts with both Vpu and Ubp, we wanted to see whether Ubp can also stably associate with membranes. Flotation gradient analysis results in the fractionation of membranes, which are relatively low in density, from other components of disrupted cells by flotation in high-percentage sucrose. Proteins that are stably associated with cellular membranes can segregate with the membrane fraction. Thus, we subjected disrupted cells to this fractionation and determined the location of Ubp in the gradient by Western analysis. In conjunction with detection of Ubp we assayed for the presence of the membrane-associated protein, 5′-nucleosidase. As shown in Fig. 5, this technique resulted in the partition of Ubp to higher density fractions of the gradient rather than to the membrane-rich fractions near the top of the gradient. These data indicate that Ubp is not stably associated with membranes.

Expression of Vpu results in cytoplasmic redistribution of Gag and Ubp

Deora *et al.* (2000) found that fusion proteins composed of the MA domain of Gag appended to green fluorescent protein were located at different intracellular locations in the presence and in the absence of Vpu. To determine whether Vpu expression affects the spatial

distribution of both Ubp and Gag we transiently expressed wild-type or *vpu*$^-$ HIV expression constructs in HeLa cells and examined the intracellular distribution of Ubp and full-length Gag. The efficiency of viral particle release was measured as a control for successful transfection. Typically, Vpu enhances particle exit from 5- to 10-fold. HIV-1 particle release was measured from these cell populations using an ELISA that quantifies intracellular and extracellular p24 antigen (Callahan *et al.,* 1998) and calculating the ratio of extra- to intracellular gag protein. These data indicate that Vpu facilitated particle exit as expected (data not shown).

Transient transfection of cells results in the successful introduction of DNA into only a subset of the cell population. However, it was possible to readily identify transfected cells and cotransfected cells simply by screening for the presence or absence of Gag protein expression. Dual detection of Gag and Ubp in transfected cells, using antibodies specific for these two proteins in conjunction with confocal imaging, revealed that both Ubp and Gag are differentially located in the absence or in the presence of Vpu. In the absence of Vpu, Ubp was distributed in the cytoplasm in a manner indistinguishable from that of untransfected cells (Fig. 6A). Moreover, Gag protein was generally localized throughout the cytoplasm; Gag appeared to be situated in an overall uniform distribution within the cytoplasm but in a large number of local centers apparently containing higher concentrations of Gag. In contrast, coexpression of Vpu with Gag resulted in a striking accumulation of Gag near the plasma membrane (Fig. 6B). Moreover, in the presence of Vpu, Ubp was also redistributed from a position overlapping with the cytoskeleton to peripheral regions coincident with Gag.

To characterize the association of Gag with the cytoskeleton, and to determine whether Vpu expression affected either cytoskeletal or membrane association of Gag, we carried out cytoskeletal extraction, as in Fig. 4, and detected Gag using Western analysis. Based on this assay, Gag appeared to be associated with both the cytoskeletal (insoluble) and the cytoplasmic (soluble) fractions (Fig. 7). Furthermore, coexpression of Vpu did not detectably affect the distribution of Gag with the cytoskeleton. To see whether Vpu affected the association of Gag with membranes, we carried out flotation gradient analysis of Gag from cells that either coexpressed or lacked Vpu. This analysis indicated that most of the Gag protein was membrane associated and that the expression of Vpu did not markedly affect the association of Gag with membranes (Fig. 8).

On the nature of association between Ubp and the cytoskeleton

Our confocal data are consistent with association of Ubp with the cytoskeletal microtubules. However, the foundation of that association is unclear since Ubp does not associate stably coimmunoprecipitate with tubulin and is segregated from the cytoskeletal fraction during *in vitro* fractionation. Further, there does not appear to be indirect interaction between Ubp and the cytoskeleton by way of cytoskeletal-associated membrane vesicles since Ubp does not stably associate with membrane fractions in membrane flotation gradients. These data suggest that the interaction between Ubp and the cytoskeleton is tenuous, regardless of whether that interaction is mediated directly or indirectly.

On the interaction between Vpu and Ubp and between Ubp and Gag

In contrast to Ubp, Vpu is tightly associated with cellular membranes, and Vpu is probably transported toward the plasma membrane by way of Golgi-derived vesicles that are trafficked along the cytoskeleton. Therefore, it is likely that Vpu and Ubp interact at the surface of these actively transported vesicles.

HIV-1 Gag protein is present in cells either in unmyristylated form in the cytoplasm or in myristylated form largely associated with cellular membranes. In the paradigm of retrovirus assembly and budding, myristylation of Gag is required for membrane association, and interaction is generally considered to occur initially and directly with the plasma membrane. Since Ubp appears to be associated with the cytoskeleton, it is not obvious how Gag and Ubp would efficiently encounter each other for efficient interaction. However, it is also possible that Gag associates with intracellular membranes. If so, then this might provide a more efficient mechanism to facilitate Ubp–Gag association. It is probably more relevant to consider the interaction and intracellular location of Ubp and Gag in the presence of Vpu since all three proteins are present in virus-infected cells. In the presence of Vpu, Ubp appears to be displaced from its normal cytoskeletal location to a position close to the cell periphery. It is significant that the intracellular distribution of Gag also appears to change in the presence of Vpu to a position that overlaps that of Ubp. This Vpu-dependent intracellular localization of both Ubp and Gag could facilitate the interaction between Ubp and Gag.

Vpu functions to promote exit of viral capsids from virus-producing cells. It seems likely that redistribution of Gag protein to a position adjacent to the plasma membrane as a consequence of Vpu expression is involved in Vpu-mediated particle exit. The mechanism by which Vpu effects this redistribution of Gag is unclear. However, the fact that Vpu and Ubp interact with each other, and that Ubp and Gag interact with each other, suggests a model in which Vpu drives the movement of Ubp from microtubules to the cell periphery. The translocation of Ubp might then result in concomitant redistribution of Gag to the cell surface.

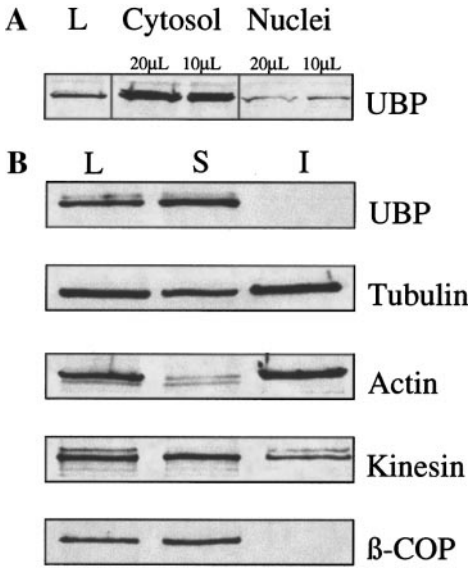

FIG. 4. Cytoplasmic, nuclear, and cytoskeletal fractionation and detection of Ubp in HeLa cells. (A) Cytoplasmic and nuclear fractions were obtained from HeLa cells and analyzed by Western blotting with affinity-purified anti-Ubp. (B) Cytoskeletal and detergent extraction of HeLa cells. L, cell lysate; S, soluble fraction (cytoplasmic); I, insoluble fraction (cytoskeleton). Actin and microtubules were stabilized and detergent extracted as described under Materials and Methods. Kinesin and β-COP were used as controls for microtubule-associated and membrane-associated proteins, respectively.

Preliminary data indicate that Ubp functions as a co-chaperone, modulating the activity of the multiprotein chaperone complex (Angeletti and Panganiban, submitted). The association of Ubp with microtubules is consistent with such a function for Ubp. It is possible that Ubp may be involved in the process of HIV-1 viral particle assembly, perhaps as a factor that helps shuttle Gag to the plasma membrane where Gag can readily assemble into capsids. Alternatively, Ubp may act as a negative factor in viral particle assembly, whereby binding to Gag inhibits the proper transport of the protein and reduces the formation of viral particles at the membrane surface. This latter hypothesis postulates that the role of Vpu is to bind Ubp and remove it from interfering with Gag transport to the plasma membrane and/or assembly into viral particles.

MATERIALS AND METHODS

Cell culture, transfection, and colchicine treatment

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Eight hundred microliters of HeLa cells at 5×10^6/ml were electroporated in 0.4-cm electrode cuvettes at 0.27 kV, 900 μF with 40 μg of plasmid DNA for immunofluorescence and with 20 μg DNA for all other experiments. For colchicine treatment, cells were incubated with 10 μg/ml colchicine (Sigma) in feeding medium for 4 h prior to immunofluorescence.

DNA constructs

G108 and BG135 have been previously described (Callahan *et al.,* 1998). Briefly, GB108 is a derivative of HIV-1 NL4-3 containing a deletion in the envelope gene. This construct is referred to as wild-type (WT) in this paper since the *env* gene is dispensable for particle formation, budding, and Vpu responsiveness. BG135 is derived from GB108 and contains an insertion in the *vpu* gene that alters the reading frame after the first 10 amino acids. This construct is referred to as Vpu$^-$ in this paper.

Immunofluorescence

HeLa cells were plated onto glass cover slides at 20×10^3/ml and incubated overnight prior to staining. Slides were prepared by being washed in ice-cold PBS 3×, fixed, and permeabilized in 100% methanol for 20 min at −20°C. Cells were then treated for 30 min at room temperature with blocking buffer (5% dry milk, 20 mM Tris, 0.01% NaN_3). For concurrent imaging of Ubp with

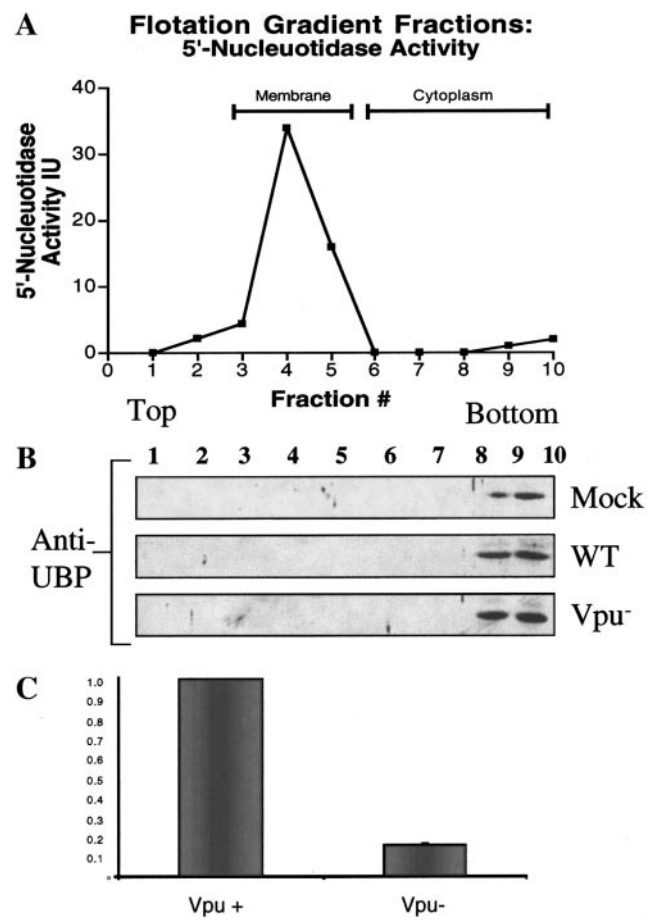

FIG. 5. Flotation gradient analysis of Ubp. Cells were sonicated and fractionated in a sucrose flotation gradient as described under Materials and Methods. (A) Measurement of 5′-nucleotidase, a plasma membrane marker, in collected fractions. (B) Western analysis of Ubp. (C) The efficiency of particle release in the presence and in the absence of Vpu.

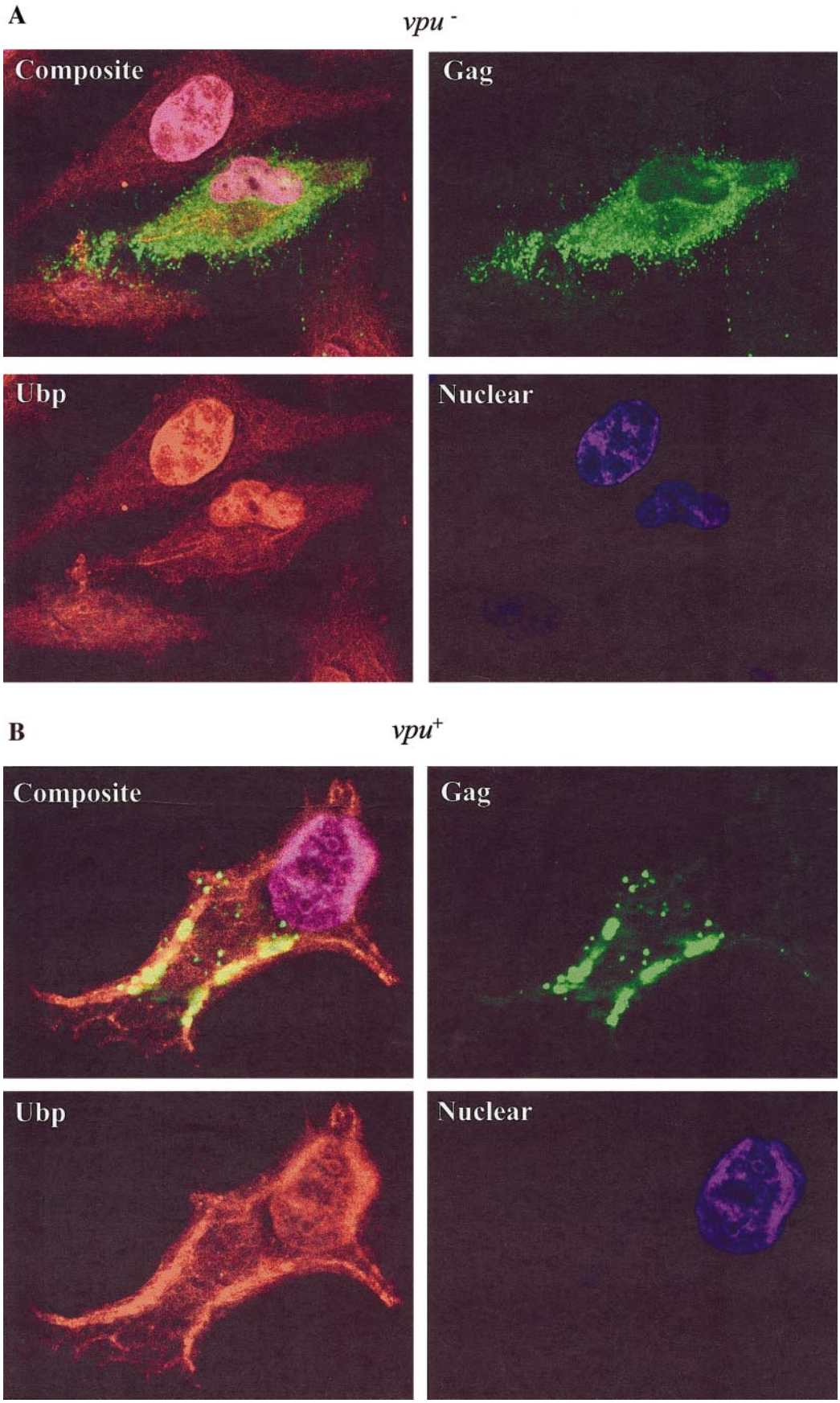

FIG. 6. Vpu-dependent redistribution of Ubp and Gag. (A) Intracellular distribution of Gag and Ubp in cells lacking Vpu. (B) Intracellular distribution of Gag and Ubp in cells expressing Vpu.

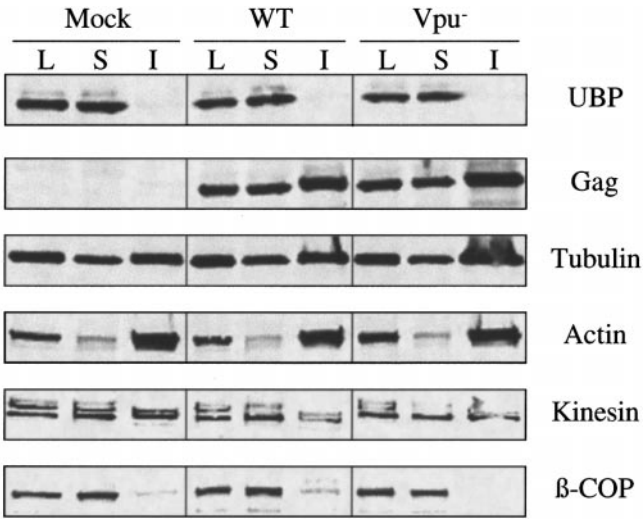

FIG. 7. Cytoskeletal fractionation of cells transfected with viral expression constructs. Annotations and abbreviations are as in Fig. 4.

tubulin or Ubp with actin, cells were incubated with rabbit anti-Ubp (antigen affinity-purified) diluted in blocking buffer for 2 h at 4°C. The slides were then washed 4× with ice-cold PBS. The cells were then incubated with TRITC-labeled goat anti-rabbit diluted at 1:200 at room temperature for 1 h. Slides were washed 4× in PBS, incubated with monoclonal FITC-conjugated anti-tubulin, 1:200, or monoclonal FITC-labeled anti-actin, 1:100, diluted in blocking buffer, and incubated at room temperature for 1 h. For Gag staining, antibody KC57-FITC was diluted 1:100 in blocking buffer and incubated at room temperature for 1 h. Slides were washed 2× with PBS, and nuclei were stained with TO-PROTM 3 iodide (Molecular Probes, T-3605) diluted at 1:10,000 and incubated for 10 min at room temperature. Cells were washed an additional 3×, mounted onto glass microscope slides with anti-fade mounting medium (Molecular Probes, P-7481), and allowed to dry overnight prior to viewing.

Confocal microscopy

Slides were imaged using a Bio-Rad MRC1024 laser scanning confocal microscope (Bio-Rad Microsciences, Hercules, CA) mounted on a Nikon Optiphot upright stand using a Nikon 60× NA 1.4 objective lens. Single optical sections and Z-series of the triple-labeled samples were imaged simultaneously using the 488-, 568-, and 647-nm lines of an air-cooled krypton–argon laser. The images were further processed using Adobe Photoshop (Paddock *et al.,* 1997).

Cytoplasmic fractionation and nuclear extraction

This method was performed as described by Cziepluch *et al.* (1998). Briefly, 5×10^6 HeLa cells were disrupted in a hypotonic solution (20 mM HEPES–KOH, pH 7.4, 7.5 mM $MgCl_2$, 0.1 mM DTT) using a tight-fitting Dounce homogenizer and incubated on ice for 20 min. The homogenate was centrifuged for 5 min at 12,000*g* and cytoplasmic supernatant was removed. The resulting nuclei-rich pellet was incubated with an equal volume of hypotonic solution adjusted to 0.5 M NaCl on ice for 30 min. The nuclear extract was then clarified by low-speed centrifugation.

Cytoskeleton isolation and detergent extraction

The method used for the isolation of the cytoskeleton was modified from those reported by Liu *et al.* (1999). HeLa cells were collected by trypsinization and normalized to 0.5×10^6 cell/ml. One milliliter of cells was pelleted, washed 1× with ice-cold PBS, then washed twice with 50 μl cytoskeleton stabilizing buffer (CSB) [100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.9) with 1 mM EGTA, Taxol, and 66 μg of phalloidin/ml]. Cells were lysed in 50 μl CSB containing 1% Triton X-100, 2 mM GTP, aprotinin (2 μg/ml), phenylmethylsulfonyl fluoride (50 μg/ml), leupeptin (2 μg/ml). Cell lysates were centrifuged at 2000*g* for 5 min and 50 μl of 2× SDS–PAGE sample buffer was added. The supernatant (cytoplasmic and detergent-soluble membrane fraction) was removed from the pellets and clarified by centrifugation at 5000*g.* The remaining pellet was washed twice with 50 μl CSB lysis buffer and solubilized in 100 μl of 1× SDS–PAGE sample buffer. Samples were heated in a boiling water bath for 3 min and run on a 10% SDS–PAGE. Gels were blotted onto PVDF membrane (Immobilon P, Millipore) and analyzed by Western analysis with anti-

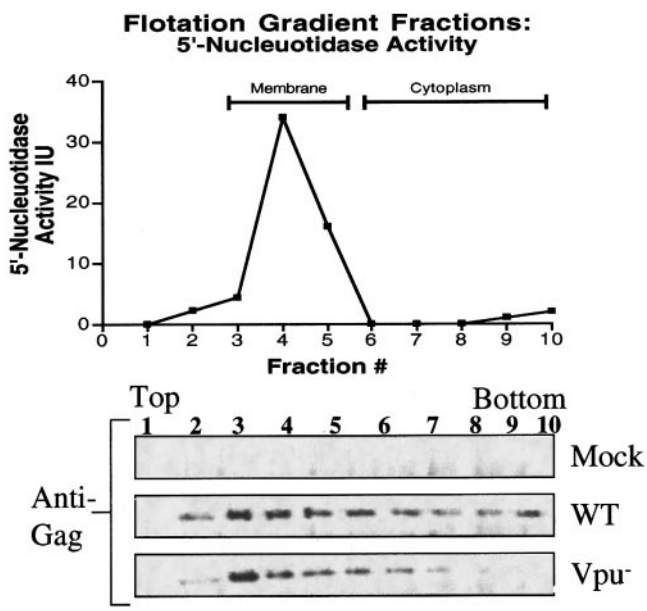

FIG. 8. Association of Gag with cellular membranes in the presence and in the absence of Vpu. Flotation gradient analysis was performed as described in the text and as described in the legend to Fig. 5.

bodies to Ubp, tubulin, actin, kinesin, or β-COP as previously described (Callahan *et al.,* 1998).

Equilibrium flotation gradient centrifugation

Flotation gradient fractionation was performed as reported by Ono and Freed (1999). HeLa cells were washed once with ice-cold PBS and collected by scraping. Transfected cells were harvested 2 days posttransfection. The cells were resuspended in 10 mM Tris–HCl (pH 7.5) containing 1 mM EDTA (TE), 10% sucrose containing aprotinin (2 μg/ml), phenylmethylsulfonyl fluoride (50 μg/ml), and leupeptin (2 μg/ml). Cells were disrupted by sonication with two 15-s bursts, in ice water. Debris and nuclei were removed by low-speed centrifugation. Two hundred fifty microliters of clarified supernatant was mixed with 1.25 ml of 85.5% (wt/vol) sucrose in TE and placed at the bottom of a centrifuge tube. Seven milliliters of 65% (wt/vol) sucrose in TE was then layered on top of the sample. An additional 3.25-ml layer of 10% (wt/vol) sucrose in TE was then added to the top of the tube. The gradients were centrifuged in a Beckman SW41T at 100,000*g* for 18 h at 4°C. Ten 1.2-ml fractions were taken from the top of the tubes and analyzed for 5′-nucleotidase activity and by Western blotting for Ubp or HIV-1 Gag protein.

Antibodies

Production and purification of anti-Ubp have been previously described (Callahan *et al.,* 1998). The anti-Ubp used in the experiments described was antigen affinity-purified. Antibodies purchased from Sigma were as follows: anti-tubulin FITC-conjugated (T 2168), anti-tubulin (T 9026), anti-actin FITC-conjugated (F 3777), anti-actin (A 2547), anti-kinesin (K 1005), anti-β-COP (G 2279).

Western blotting

Samples were mixed with standard SDS–PAGE protein sample buffer and subjected to electrophoresis on a 10 or 12% SDS polyacrylamide gel. Proteins were transferred to an Immobilon-P membrane (Millipore) in transfer buffer (27.2 mM Tris, 192 mM glycine, 20% methanol). Membranes were air-dried at room temperature for 1 h and then incubated with blocking buffer (5% dry milk, 20 mM Tris, 0.01% NaN_3) containing primary antibodies overnight at 4°C. Membranes were washed three times in wash buffer (20 mM Tris, 100 mM NaCl, 0.3% Tween 20, 0.05% NaN_3) and incubated with secondary antibodies conjugated with alkaline phosphatase for 2 h at room temperature. Membranes were washed three times with wash buffer and developed with 5-bromo-4-chloro-3-indolyphosphate-nitroblue tetrazolium (Sigma) for 15 min at room temperature.

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